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Award Number: DAMD17-98-1-8299

TITLE: Chemotherapy of Late Stage Breast Cancer Targeted Towards Cell Cycle Regulatory Components

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REPORT DATE: October 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | October 2001 | Annual (15 Sep 00 - 14 Sep 01) |

**4. TITLE AND SUBTITLE**
Chemotherapy of Late Stage Breast Cancer Targeted Towards Cell Cycle Regulatory Components

**5. FUNDING NUMBERS**
DAMD17-98-1-8299

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**
Report contains color

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 Words)*

Two drugs have been studied as potential therapeutic agents for late stage breast cancer patients. 5-aza-2'deoxycytidine (5-Aza-CdR), is an inhibitor of DNA methylation and is targeted for re-expression of a repressed tumor suppressor gene known as p16. Re-activation of the p16 gene in cells in which the gene is methylated will restore normal growth control and be efficacious in treatment of breast cancer patients. Both p16 protein expression and de-methylation of the p16 promoter occurs in breast cancer cells, treated with 5-Aza-CdR. 5-Aza-CdR also destroys the ability of the cells to grow in an anchorage-dependent manner, thereby indicating that the drug can inhibit tumorigenesis. We have currently developed a better model for the action of the drug by screening a number of breast cancer cell lines for methylated p16 and then testing the efficacy of the drug in a nude mouse model. A non-invasive blood test has also been developed for the detection of p16 methylation that will allow us to easily identify patients suitable for a 5-Aza-CdR clinical trial. Clinical protocols to accomplish this goal have been approved. A phase II clinical trial with a second drug, Bryostatin-1, has been opened again, but no patients have yet been accrued.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| Breast Cancer, CTR, Chemotherapy | 42 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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## Introduction

During the past decade a very large increase in our knowledge of biological mechanisms regulating progression of cells through the cell division cycle has taken place. Together with the development of this knowledge, it has been shown by work in many laboratories that essentially all cancer cells have one or more defects in the components known to regulate cell cycle progression. For example, our recently completed studies of cell cycle regulatory defects in breast cancer cells, carried out with a grant from the Army Breast Cancer Research Program, showed that loss of expression of the cyclin-dependent kinase inhibitor p16, sometimes accompanied by overexpression of cyclin D1, is a common defect in breast cancer cells. These findings, plus the large amount of work carried out by others, presented a new potential target for cancer chemotherapy. Our proposal to exploit such targets for the chemotherapy of breast cancer is the basis for the current Clinical Translational Research Grant. We proposed to explore two drugs known or expected to cause changes in the expression of cell cycle regulatory components as potential chemotherapeutic agents in the treatment of late stage breast cancer. Bryostatin-1, shown by Kraft and coworkers to cause increases in the expression of the cyclin-dependent kinase inhibitor p21, was chosen as an agent to be tested in a phase II clinical trial. 5,6-dihydro-5-azacytidine, a DNA methylation inhibitor with less toxicity than the commonly studied 5-aza-2'-deoxycytidine, was chosen for pre-clinical studies directed towards eventually implementing a phase II clinical trial of that drug. DNA methylase inhibitors have been shown to increase the expression of p16 protein in cells where lack of expression is due to methylation of the p16 gene. The results of the third year of the grant are reported here.

# Body of Report

## Materials and Methods

Breast cancer cell lines and tumor material

The breast cancer cell lines T47D-DE and T47D-CO were obtained from the University of Colorado Tissue Culture Core Facility and 20 other breast cancer cell lines (listed in **Table 1**) were obtained from the American Type Culture Collection. Cell lines were cultured according to directions from the ATCC. A list of different cell culture media requirements for each individual cell line is shown in **Table 2**.

Antibodies

The anti-p16 was obtained from Oncogene. The horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad.

Protein extraction and western blot analysis

Cells were harvested and washed in PBS then resuspended in Laemmli sample buffer (Laemmli, 1970). After boiling for 4 minutes, the extracts were sheared through a 26-gauge syringe needle, aliquoted, and stored at -80°C.

Approximately 100 mg of each protein extract were subjected to SDS/PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes (Schleicher and Schuell) for 45 minutes at 0.45 A using the Genie Electrophoretic Blotter (Idea Scientific, Minneapolis). Membranes were stained with Ponceau dye to control for equal loading, and immunodetection performed using the enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer's instructions.

Isolation of DNA from cell lines and blood plasma

Cell line DNA was isolated by incubating cells at 55°C in lysis buffer (10 mM Tris pH 8.0, 2.0 mM EDTA pH 8.0, 10 mM NaCl, 5% SDS) containing 1 mg/ml Proteinase K. The samples were then subjected to two phenol-chloroform extractions and one chloroform:isoamylalcohol (24:1) extraction followed by ethanol precipitation.

To isolate plasma DNA, 10 ml plasma were first heated to 99°C for 5 minutes then centrifuged at 14 K rpm. The clear supernatant was incubated overnight in one-tenth volume of 20 mg/ml Proteinase K (in double distilled water) and one-tenth volume AL buffer (Qiamp Blood Kit, Qiagen Inc., Hilden, Germany) after which the DNA was purified on QIAamp columns (Qiamp Blood Kit, Qiagen Inc., Hilden, Germany)

according to the 'Blood and Body Fluids protocol'. The DNA was eluted from the column with 200ul double distilled water.

DNA analysis by Methylation-specific PCR (MSP)

Two micrograms of cell line DNA or one-fourth of the total plasma DNA sample was modified with sodium bisulfite using a modified method of (Herman et al., 1996) kindly sent to us by S. Belinsky (University of New Mexico). The DNA was then precipitated with ammonium acetate (3M final concentration) and two volumes of ethanol. The resulting templates were subjected to a nested, two-stage PCR. This two-step PCR approach improves the sensitivity to detect methylated alleles by >50 fold over the original method (Palminsano et al. 2000). Templates were subjected to stage-1 PCR with primers designed from the promoter of the p16 gene (Palminsano et al. 2000). A 50$\mu$l reaction mixture contained a final concentration of 10$\mu$M of each oligonucleotide, PCR buffer supplied by Qiagen Hotstar Taq DNA polymerase kit (10x Buffer= Tris.Cl, KCl, $(NH4)_2SO4$, 15mM $MgCl_2$, pH8.7 note:Qiagen does not describe specific concentrations for its buffer); 200 mM dNTPs; and 1.25 units Qiagen Hotstar Taq DNA polymerase. The DNA was subjected to 40 cycles of amplification consisting of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and elongation for 30 seconds at 72°C, followed by a final elongation step of 10 minutes at 72°C. The stage-1 PCR products were diluted 50 fold and 5$\mu$l was subjected to a stage-2 PCR. A 20$\mu$l reaction mixture contained a final concentration of 10$\mu$M of each oligonucleotide, PCR Master mix supplied by Qiagen Hotstar Taq DNA polymerase kit (Tris.Cl, KCl,$(NH4)_2SO4$, 15mM MgCl, ph8.7, 200 mM dNTPs); and 0.5 units Qiagen Hotstar Taq DNA polymerase. The DNA was subjected to 40 cycles of amplification consisting of denaturation for 15 seconds at 94°C, annealing for 15 seconds at 68°C for unmethylated-specific and methylated-specific oligonucleotides (Herman et al. 1996), and elongation for 15 seconds at 72°C, followed by a final elongation step of 10 minutes at 72°C. The PCR products were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized with UV light. As described in the Results section of the Statement of Work, multiple steps were required to prevent cross-contamination of PCR samples. Some of these steps included: (1) filter pipette tips were used for all PCR reactions, (2) filter sterilized H2O was used instead of autoclaved H2O, (3) we reduced the number of pipette manipulations by creating a "mastermix" of all the components required for PCR except for the DNA template and Taq polymerase, (4) "mastermix " components were stored in a freezer away from DNA templates, and (5) DNA template was added to reactions as the final step in setting up the reactions.

Tumorigenicity Studies of Human Breast Cancer Cell Lines in Athymic Nude mice.

The growth of human breast cancer cell line T47D-DE in nude mice was tested with xenographs in animals treated or not treated by repeated intraperitoneal administration of the drug 5-Aza-CdR or with no drug (PBS control), by injecting subcutaneously on the right and left flank of each animal with a suspension (volume 0.4ml) of growth factor reduced Metragel (BD Biosciences) and living human breast

cancer cells that were grown previously in tissue culture. The cell line T47D-DE was tested in mice that have been ovarectomized and hormone replacement accomplished via insertion of a time-release (60 day) estrogen (17Beta-estradiol, Innovative Research of America) hormone release pellet inserted at the base of the mouse's neck. The mice were handled exclusively in the UCHSC Animal Resource center, using sterile equipment, clothing, and facilities. They were maintained there after surgery and injections, and inspected weekly to measure the development of and size of any tumors. After a period of not more than 4 weeks we injected the mice three times a week at a dose of 3mg/kg of the drug 5-Aza-CdR or sterile PBS (control) for an additional 4 weeks. The mice were sacrificed humanely by $CO_2$ narcosis and any tumor removed for molecular and pathological analysis.

## Relationship to Statement of Work

Tasks 1, 2, and 3. To implement and evaluate a Phase II clinical trial designed to test the chemotherapeutic efficacy of bryostatin 1 in Stage IV breast cancer patients who have failed high dose chemotherapy.

Bryostatin-1 Trial for Breast Cancer

Because of the closure of the IRB over the last year we have not been able to recruit any patients to this trial. After the IRB closure was ended the protocol needed to be resubmitted. We have completed this process and now have approval.

We had a big boost in interest in this protocol, when one of the major networks aired a story showing how bryostatin-1 had an effect on inhibiting tumor growth in a patient at Sloan Kettering. This led to 50 calls from all over the world to receive this drug. Unfortunately, the majority of these patients did not fit the trial or did not realize that they would have to take the compound once a week for eight weeks, necessitating them to stay in Denver. This suggests that the trial may have to be modified to recruit patients in distant and local areas.

Task 4. To determine the effects of DHAC and 5aza-2'deoxycytidine on p16 levels and on growth and tumor formation by breast cancer cells.

As described in previous progress reports, DHAC proved to be unsuitable as a DNA methylation inhibitor capable of inducing expression of p16 in breast cancer cell lines. Thus, we turned to the more widely studied methylation inhibitor 5aza-2'deoxycytidine (5-Aza-CdR). In the previous progress report we had demonstrated that 5-Aza-CdR causes demethylation of p16 DNA and expression of p16 protein in several breast cancer cell lines including T47D-DE and T47D-CO, with concomitant loss of

growth of these cells in soft agar. To continue our studies, we proposed to test the effect of the drug 5-Aza-CdR in nude mice following tumor implantation of breast cancer cells.

Screening of Breast Cancer Cell lines for Rb(+) expression and p16(-) expression due to promoter methylation for use in tumorigenecity assays.

We decided to screen additional breast cancer cell lines with the aim of identifying cell lines that, like T47D, express functional Rb protein but lack p16 expression due to DNA promoter methylation. Our studies (shown in **Table 1**) identified two potential cell lines that could be used for tumor implantation experiments. Twenty breast cancer cell lines were obtained from the ATCC, grown in tissue culture media, and protein extracts were prepared for immunoblot analysis of both Rb and p16 proteins. Cell lines that exhibited both strong positive Rb expression and were negative for p16 expression, were further subjected to methylation-specific PCR (MS-PCR) to determine if lack of p16 expression was due to promoter methylation. Two cell lines were identified that demonstrated these criteria: HCC 202 and MDA-MB-134 VI. HCC 202 grew very poorly in tissue culture and was unable to generate enough cells for tumor injections into mice. MDA-MB-134 VI was selected as a potential cell line for tumor injection experiments. (see results below)

Tumorigenecity Pilot Experiment.

In the course of setting up our tumor experiments, we learned from experiments done in Kathryn Horwitz's laboratory (CU Health Sciences Center) that breast cancer cell line T47D would form palpable tumors in nude mice if female mice had been both previously ovarectomized and 17Beta-estradiol (estrogen) was replaced in the form of a surgically implanted time-released pellet (60-day) under the mouse's skin. Our initial pilot experiment was performed to determine if T47D and MDA-MB-134 VI cell lines could generate palpable tumors in nude mice. Several T47D cell lines are currently known to circulate. So, for our initial experiments, two T47D cell lines designated T47D-CO and T47D-DE were used. Five mice were injected with $5x10^6$ cells each along with growth factor reduced Metragel cells as a solid support for each particular cell line. The results are shown in **Figure 1**. Cell line T47D-DE was able to form tumors of relatively large size by 60 days. In addition, one mouse that was not supplemented with estrogen and another mouse that received a partial estrogen pellet were unable to form tumors demonstrating that the estrogen was required for tumor growth. Mouse #2 died at 51 days. Note that tumor sizes were followed for the T47D-DE cell line out to 112 days, much past the 60-day time-release pellet of estrogen. In two cases, tumors still experienced an increase in growth in the absence of estrogen suggesting that estrogen is required to establish tumor formation in this cell line but it is probably not required for maintenance of tumor growth.

At the same time, T47D-CO was unable to form tumors of appreciable size regardless of the presence or absence of the estrogen pellet (see **Figure 1**). Because these mice failed to form tumors of considerable size as compared to T47D-DE, T47D-CO was not studied further and hence, the experiment was terminated at 51 days.

The results of tumor growth of MDA-MB-134 VI in nude mice are shown in **Figure 2**. Although two of the mice were able to generate small tumors, the phenotypic appearance of the two tumors was non-fibrous and "water-like" unlike the hard fibrous tumors generated by T47D-DE cell line. In addition, since none of the tumors appeared to grow larger after 43 days, this experiment was also terminated.

Based on the results from this particular pilot experiment, we concluded that T47D-DE could form tumors in nude mice. Therefore, these tumors derived from T47D-DE would be of significant size to test the chemotherapeutic effects of 5-Aza-CdR. This cell line is the one that we had previously shown to contain methylated p16 that could be reactivated by 5-Aza-CdR in vitro.

5-Aza-CdR treatment of T47D-DE cell line tumors in nude mice.

Prior to testing the effects of 5-Aza-CdR as a chemotherapeutic, we wanted to determine the possible toxicity of this drug on mice. 5-Aza-CdR was injected via the intraperitoneal route into mice at three doses that had previously been determined to be non-toxic: 0.3 mg/kg, 1.0mg/kg, and 3.0mg/ kg three times per week (Jacson-Grusby et al., 1997). This experiment was conducted for one week. Of the mice that were tested, none demonstrated any particular ill effect from any of the doses. Thus, we decided for our next experiment that the highest dose of 5-Aza-CdR would be used.

The procedure for testing 5-Aza-CdR on T47D-DE in nude mice involved multiple steps. First, we generated 25 mice that contained two tumors each derived from the T47D-DE cell line by injecting $5x10^6$ cells along with the Metragel support into mice that had been previously ovarectomized and surgically implanted with 60-day timed-release estrogen pellet. Next, the mice were split into two groups: a control group of 12 mice that would be injected with phosphate-buffered saline (PBS) and an experimental group of 13 mice that would be injected with 5-Aza-CdR. In this particular experiment, two tumors were implanted into each individual mouse: one into of the left and right flank of the animal. Tumors were allowed to grow for four +1/2 weeks (33 days) prior to chemotherapy treatment. Mice were then subjected to injections via the intraperitoneal route 3 times/week to either PBS or 5-Aza-CdR treatment at the highest dose of 3.0kg/mg three times per week. Tumors were measured throughout the experiment once per week.

The results of our experiment are shown in **Figure 3** and **Figure 4**. The graphs shown demonstrate the average tumor size for the two tumors of each individual mouse. Of the 13 mice that were injected with 5-Aza-CdR, only two survived through the full term of the experiment (**Figure 3**). It became apparent that the nude mice could not tolerate the high dose of 5-Aza-CdR as most of the mice died before the end of the experiment. Additionally, they showed signs of not tolerating the drug well, because many of the mice started to lose weight rapidly. At the same time, only 6 of the 12 mice that were injected with PBS survived (**Figure 4**). It is not clear why so many of the PBS injected mice also died.

The average size of tumor for the PBS injected mice was compared to all the 5-Aza-CdR injected mice is shown in **Figure 5**. The average size of the tumor over time is smaller in the 5Aza-Cdr injected mice but appears to get smaller after the 33rd day when the injections started as compared to the PBS mice. The PBS injected mice, on the other hand, demonstrated a steady increase in tumor size over time even after control injections of PBS were begun. Although these results appear encouraging, the major limitation of this experiment is that small numbers of animals that made it through the time course of this experiment. Clearly, more animals will be required to show a statistical significance of the efficacy of the drug 5-Aza-CdR. Our future directions will concentrate on using a smaller dose of 5-Aza-CdR due to its toxic nature and hopefully increasing the number of animals that make it through the trial. Tumors isolated from these mice at termination of the experiment await characterization of the methylation status of the p16 DNA.

Task 5. To test the effects of combination of DHAC, 5-Aza-CdR and bryostatin 1 on growth and tumor formation by breast cancer cells. Months 24-48.

Methylation Inhibitors plus Bryostatin-1

A goal of this grant was to attempt to combine methylation inhibitors with bryostatin in the treatment of breast cancer. We have attempted to determine which patients have methylated p16 gene using a sensitive PCR assay on the blood and tissue samples (see Task 9 below). This will enable us to test patients that already carry the diagnosis of breast cancer for this abnormality.

We have developed two protocols to evaluate the methylation status of the p16 gene in human samples. These protocols involve a unique assay of methylated P16 DNA done using DNA that is free in the blood of patients. The first protocol 00-848 enables us to examine blood from patients that are known to have metastatic breast cancer. Patients with known metastatic disease have a single sample of blood drawn and the plasma frozen for later analysis. We predict to complete 100 samples. Statistical analysis demonstrates that p16 should be methylated in 30% of the tumors. This number of samples should give us sufficient numbers of samples to demonstrate whether this blood test is sufficiently sensitive to detect p16 DNA in the blood. We have collected seven samples of blood.

The second protocol will enable us to correlate the p16 methylation in samples taken at the time of breast biopsy or surgery with the blood samples. DNA is extracted from the tissue and the blood and then analyzed by sensitive PCR for the methylation of p16. We have received ten numbers of samples from this trial. Both protocols are under review for the next year and should be approved.

Task 6. To design a Phase II clinical trial of 5-Aza-CdR.

Task 7. To implement a Phase II clinical trial of 5-Aza-CdR in breast cancer patients who have failed high dose chemotherapy and whose tumors contain methylated p16 DNA.

Task 8. To evaluate the outcome of 5-Aza-CdR chemotherapy in terms of effects on tumor response as well as on methylation of tumor p16 DNA, expression of p16, cdk4/cdk6 kinase activity and phosphorylation state of Rb protein.

Task 9. To develop methodology for determining the p16 methylation status of breast cancer patients by measurements on plasma DNA, and to employ this methodology for the selection of patients for clinical trials of 5-Aza-CdR in breast cancer.

Analysis of Plasma DNA from breast cancer patients for breast tumor-specific methylation of the p16 gene.

One of the major objectives of the current project is to identify women whose tumors contain methylated p16 genes for recruitment into a clinical trial that aims to test the efficacy of therapeutic demethylating agents such as 5-Aza-CdR.

Several years ago, it was determined that the plasma component of circulating blood contained tiny quantities of free DNA. The concentration of DNA in the plasma of individuals diagnosed with different types of cancer approaches 180 ng/ml (Leon et al., 1977), whereas, in healthy individuals it is approximately 14 ng/ml (Shapiro et al., 1996). In our previous report, we presented data that examined the ability to detect tumor-specific defects in circulating blood. We had optimized a procedure for isolating DNA from plasma samples and showed that the resulting DNA could be successfully amplified at the p16 locus by methlyation-specific PCR. In addition, our previous experiments could detect as little as 12.5 ng of DNA in the circulating plasma.

We decided to extend this MS-PCR procedure to the blood plasma of actual breast cancer patients to validate our assay. Our initial results were complicated by the fact that the MS-PCR is a very sensitive assay, and we obtained positive PCR signals from negative control samples that did not contain any DNA template (see **Figure 6**, 9-28-00 blanks sample labeled U). In addition, the variability of the signals from one week to the next was a cause for concern. For example, patient # 9 demonstrated first signals for both unmethlyated (U) and methylated (M) during the week of 9-28-00 when this assay was first performed. During the week of 10-24-00 patient #9 only shows a MS-PCR signal for methylated (M) p16. Then, when the assay was repeated one day later, patient #9 demonstrated a signal for the (U). We feared possible cross contamination in our PCR reactions, but did not know the source of the contamination. Prior to using any more precious plasma samples from breast cancer patients, we decided to analyze the source of the DNA template contamination problem.

One example of the type of troubleshooting experiment is shown in **Figure 7**. The results of this particular experiment show that PCR signals were being generated from a negative control samples regardless of whether the PCR reactions were set up in a laboratory flow hood or on a laboratory bench.

Multiple steps were taken, over several months, to eliminate spurious PCR signals from negative control samples and include a list of the following actions that were taken:

1. Filter pipette tips used for all PCR reactions.
2. A Hotstart Taq polymerase was used to eliminate "primer-dimer" effects or non-specific priming events during the initial stages of the PCR cycles. This polymerase only becomes active when the DNA templates become denatured at 95 degrees.
3. Filter sterilized $H_2O$ was used instead of autoclaved water. The autoclave generates aerosols that could include minute traces of DNA.
4. We reduced the number of pipette manipulations by creating a "mastermix" of all the components required for the PCR except for the DNA template and Taq polymerase.
5. The "mastermix" components were stored in a freezer away from any DNA template samples. This prevented cross contamination by sample handling.
6. The DNA templates for PCR were always added to the reaction as the last step.
7. Positive control samples were generated for the methylated and unmethylated forms of the p16 gene by treating HBL100 cell line DNA with or without SssI methylase in vitro. This allowed us to test if the assays were specific for the unmethylated (U) and methylated (M) alleles of p16.

The most important thing we did was incorporate a nested, two stage PCR approach, which improves the sensitivity to detect methylated alleles by >50 fold over the original method (Palmisano et al., 2000). Basically, DNA is treated with sodium bisulfite as before to modify the DNA of methylated alleles. Then, DNA primers are used to amplify p16 that recognize the bisulfite–modified DNA template but do not discriminate between methylated and unmethylated alleles that are located outside of the region outside of the methylation-specific and unmethylation-specific primers. Once this DNA is amplified, it is then subjected to a set of nested primers that can discriminate between the methylated and unmethylated alleles.

The result of using the two-stage PCR approach and incorporating all these steps is that we eliminated the source of DNA template contamination, and at the same time decreased the amount of precious DNA template derived from patient's plasma that we had to use. The result of one of our PCR assays is shown in **Figure 8**. Of the eleven patient samples that were subjected to this two-stage MS-PCR approach, one patient demonstrated a methylated p16 allele. This result has since been confirmed, and the tumor tissue sample that came from this patient awaits characterization of p16 expression by immunoblot analysis and p16 methylation by MS-PCR. Both the positive and negative controls for this particular experiment were clean. We have screened a total of 21 patient

Samples (19 frozen samples from 1990-1992, and 2 new samples from the clinical protocol 2000-2001) and so far only one patient has been shown to demonstrate a methylated p16 allele.

Thus, we conclude that the two stage nested MS-PCR approach can be used to detect methylated alleles of p16 from breast cancer patients. Our future experiments will be directed at analyzing more patient samples prior to submitting patients to chemotherapeutic trials.

## Key Research Accomplishments

- We have identified two new breast cancer cell lines that are methylated at the p16 locus that fail to express p16 protein: HCC202 and MDA-MB-134 VI.

- In addition, we have developed a model that will allow us to study human breast cancer tumors in nude mice. We are now using this model system to test the efficacy of methylation inhibitors such as 5-Aza-CdR in slowing or reducing tumor growth by reestablishing expression of p16 in T47D-DE tumor cells.

- A non-invasive method for determining p16 methylation status in breast cancer patients has been developed utilizing MS-PCR. Methylated p16 sequences are readily detectable in DNA isolated from plasma of patients. So far, one patient out of 21 screened has been identified harboring a methylated p16 allele.

## Reportable Outcomes

There have been no reportable outcomes to date.

## Conclusions

Our previous studies of the effects of 5Aza-Cdr on p16 gene methylation and p16 protein expression in breast cancer cell lines have shown that this drug effectively blocks p16 gene methylation and induces p16 protein production. 5-Aza-Cdr also blocks anchorage-independent growth of breast cancer cell lines, indicating that treatment with this drug abrogates the tumorigenic properties of these cells. However, control experiments employing breast cancer cell lines with deleted p16 genes, or which fail to express Rb, also lose the ability to grow in an anchorage-independent manner when treated with 5-Aza-Cdr, indicating that effects on gene expression other than the induction of p16 may contribute to this effect. Therefore, in our current studies, we have developed a better model for the action of the drug. We accomplished this goal by screening a number of breast cancer cell lines to find ones that have both methylated p16 and can produce tumors in nude mice to test the efficacy of the drug in a more physiological situation. Our preliminary results suggest that 5-Aza-CdR may reduce the size and/or growth of methylated p16 breast cancer cell line T47D-DE derived tumors in nude mice. However, this experiment needs to be repeated to generate statistical numbers of mice that make it through the trial.

Our development of a protocol for detecting methylated p16 sequences in plasma DNA from breast cancer patients provides a potential non-invasive procedure for determining the p16 methylation status of breast cancer patients. These results have led us to propose and design a clinical protocol to determine whether plasma DNA measurements of methylated p16 sequences are a reliable indicator of the p16 methylation status of breast tumor DNA. This methodology allows investigation of p16 DNA methylation in patients with advanced metastatic breast cancer, since many of these patients are not suitable for biopsy procedures.

Because of the closure of the IRB over the last year we have not been able to recruit any patients to the Bryostatin-1 trial. After the closure was ended, the protocol needed to be resubmitted. We have completed this process and now have approval.

We had a big boost in interest in this protocol, when one of the major networks aired a story showing how bryostatin-1 had an effect on inhibiting tumor growth in a patient at Sloan Kettering. This led to 50 calls from all over the world to receive this drug. Unfortunately, the majority of these patients did not fit the trial or did not realize that they would have to take the compound once a week for eight weeks, necessitating them to stay in Denver. This suggests that the trial may have to be modified to recruit patients in distant and local areas.

**"So What" Section**

We believe that by combining clinical and basic science studies we can identify new therapies for patients with late stage breast cancer. For example, we have developed a physiological mouse model system to determine the efficacy of methylation inhibitors on human breast cancer tumors. These inhibitors could be used in the clinic in patients that have methylated p16. We have also developed a blood test to identify these patients.

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# Appendix

Figures 1 to 8

Tables 1 and 2

Two Clinical Protocols and Patient Consent Forms:

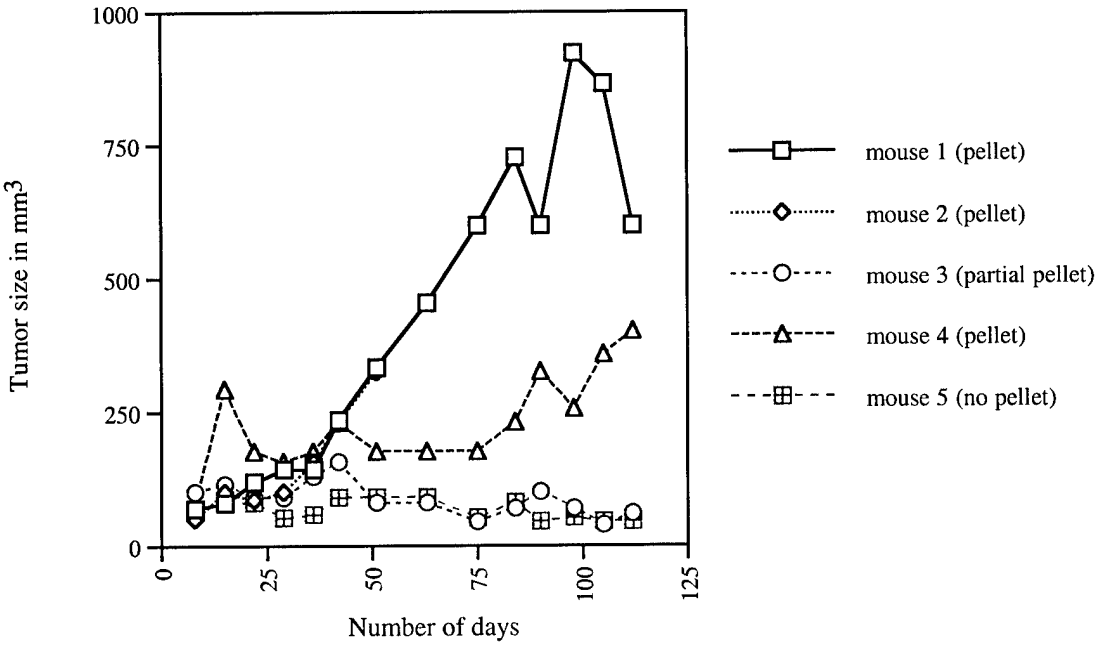
#00-848: "Estimation of abnormal breast cancer DNA in patient's blood using a sensitive PCR based assay"

#00-849: "Correlation between methylated p16 DNA in the blood and breast cancer tissue of patients"

**Figure 1. Analysis of Tumor Growth by Breast Cancer Cell Lines T47D-DE and T47D-CO Injected into Nude Mice.**

Breast Cancer Cell lines T47D-DE and T47D-CO were grown in tissue culture prior to injecting a total of $5x10^6$ cells into the flank of nude mice along with the solid support Metragel. The mice had been ovarectomized and human estrogen (17-Beta-estradiol) was surgically implanted under the skin of the neck of the mouse as a time-released pellet (60 day). Plotted are the sizes of the tumor in $mm^3$ versus time in number of days. Note that some of the individual mice received an estrogen pellet, whereas some did not. This is displayed in the legend to the right of the graphs. Also, both graphs are plotted on the same scale for direct comparison.

T47D-DE Tumor Growth in Nude Mice




T47D-CO Tumor Growth in Nude Mice

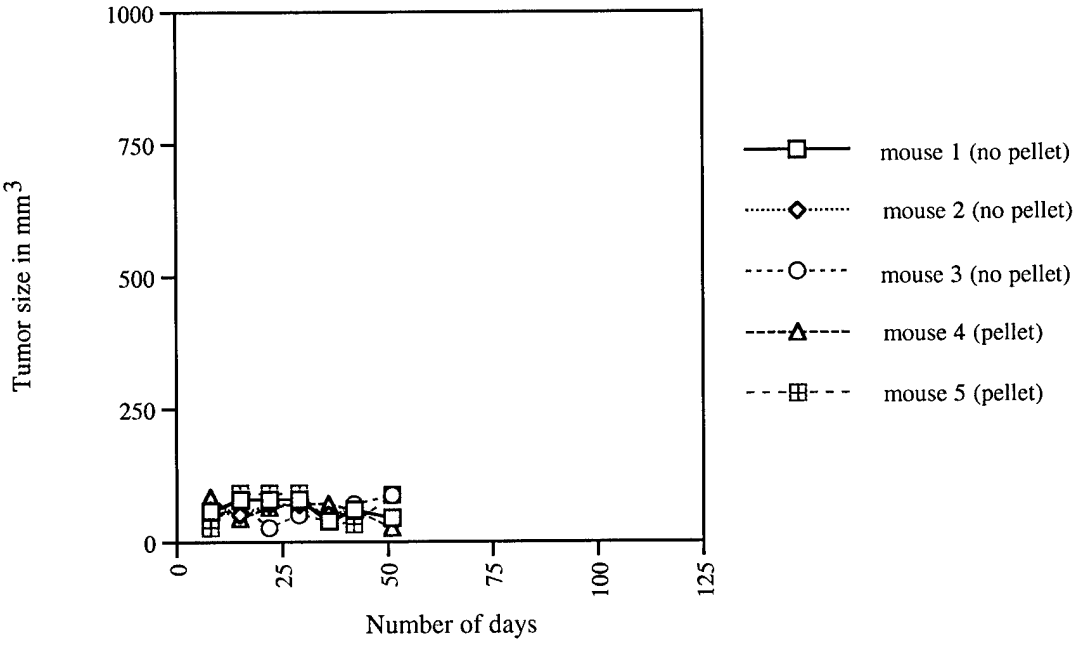


**Figure 2. Analysis of Tumor Growth by Breast Cancer Cell MDA-MB-134 VI Injected into Nude Mice.**

Breast Cancer Cell MBA-MB-134 VI was grown in tissue culture prior to injecting a total of $5x10^6$ cells into the flank of nude mice along with the solid support Metragel. The mice had been previously ovarectomized and human estrogen (17-Beta-estradiol) was surgically implanted under the skin of the neck of the mouse as a time-released pellet (60 day). Plotted are the sizes of the tumor in $mm^3$ versus time in number of days.

MDA-MB-134 VI Tumor Growth in Nude Mice

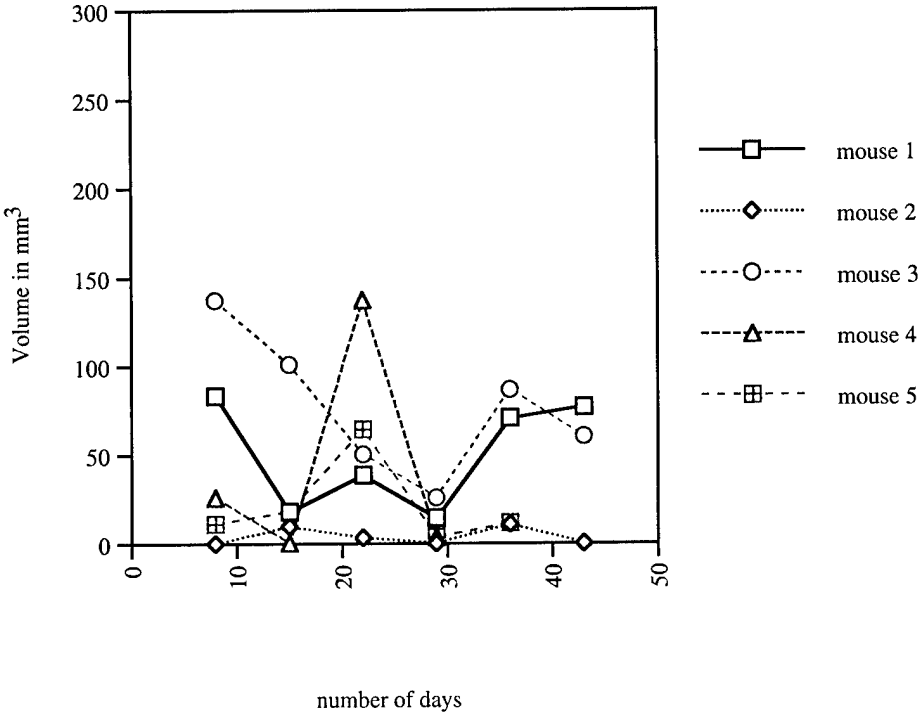


**Figure 3. T47D-DE Tumor Growth in 5-Aza-CdR Injected Nude Mice.**

Breast Cancer Cell line T47D-DE was grown in tissue culture prior to injecting a total of $5x10^6$ cells each into both the right and left flanks of nude mice along with the solid support Metragel. The mice had been previously ovarectomized and human estrogen (17-Beta-estradiol) was surgically implanted under the skin of the neck of the mouse as a time-released pellet (60 day). Plotted are the average sizes of the tumors in $mm^3$ for each individual mouse versus time in number of days. Tumors were allowed to grow for 4 and 1/2 weeks (33 days) prior to starting the chemotherapeutic 5-Aza-CdR injections. 5-Aza-CdR was injected via the intraperitoneal route at a dose of 3.0 mg/kg in a volume of 0.1ml three times weekly. Tumor sizes were measured weekly throughout the course of the experiment. Of the total of thirteen nude mice that were used, only two survived to the completion of the experiment. This graph shows only the data for the two mice that made it to completion.

T47D-DE Tumor Growth in 5-Aza-Cdr Injected Nude Mice

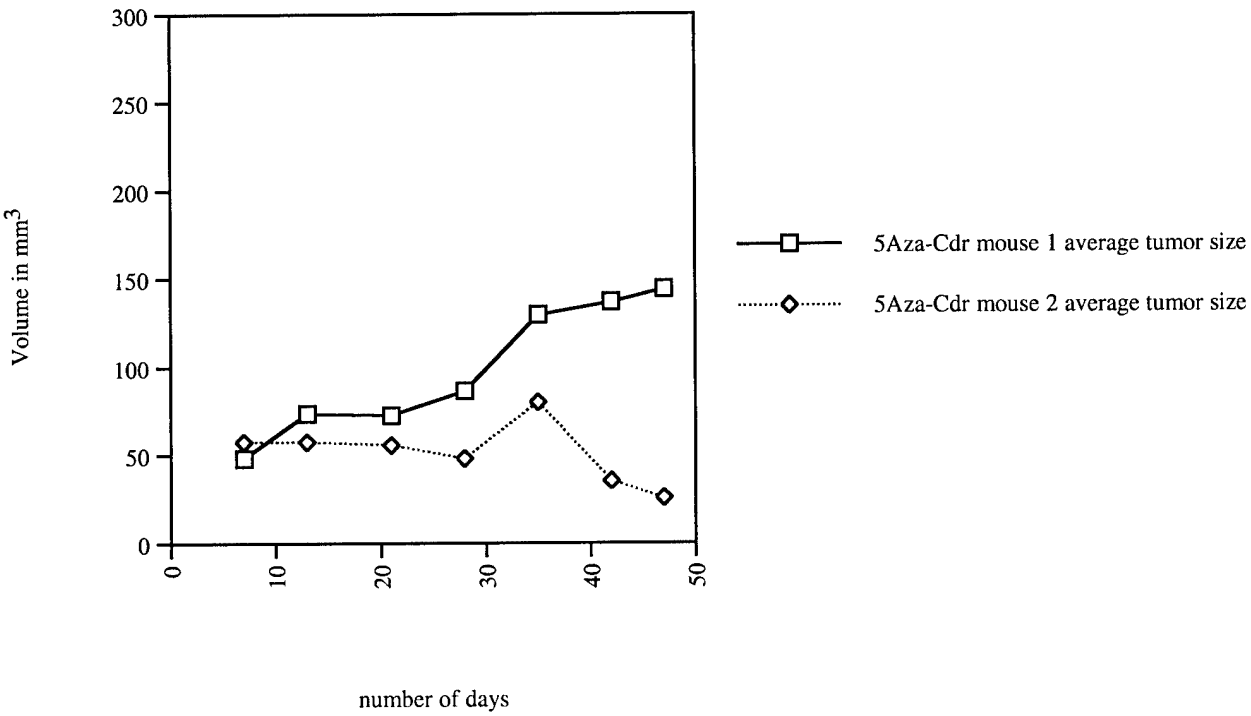


**Figure 4. T47D-DE Tumor Growth in Control PBS-Injected Nude Mice.**

Breast Cancer Cell line T47D-DE was grown in tissue culture prior to injecting a total of $5x10^6$ cells each into both the right and left flanks of nude mice along with the solid support Metragel. The mice had been previously ovarectomized and human estrogen (17-Beta-estradiol) was surgically implanted under the skin of the neck of the mouse as a time-released pellet (60 day). Plotted are the average sizes of the tumors in $mm^3$ for each individual mouse versus time in number of days. Tumors were allowed to grow for 4 and 1/2 weeks (33 days) prior to starting the control phosphate-buffered-saline (PBS) injections. PBS was injected via the intrperitoneal route in a volume of 0.1ml three times weekly. Tumor sizes were measured weekly throughout the course of the experiment. Of the total of twelve nude mice that were used, only six survived to the completion of the experiment. This graph shows only the data for the six mice that made it to completion.

T47D-DE Tumor Growth in PBS-injected Nude Mice

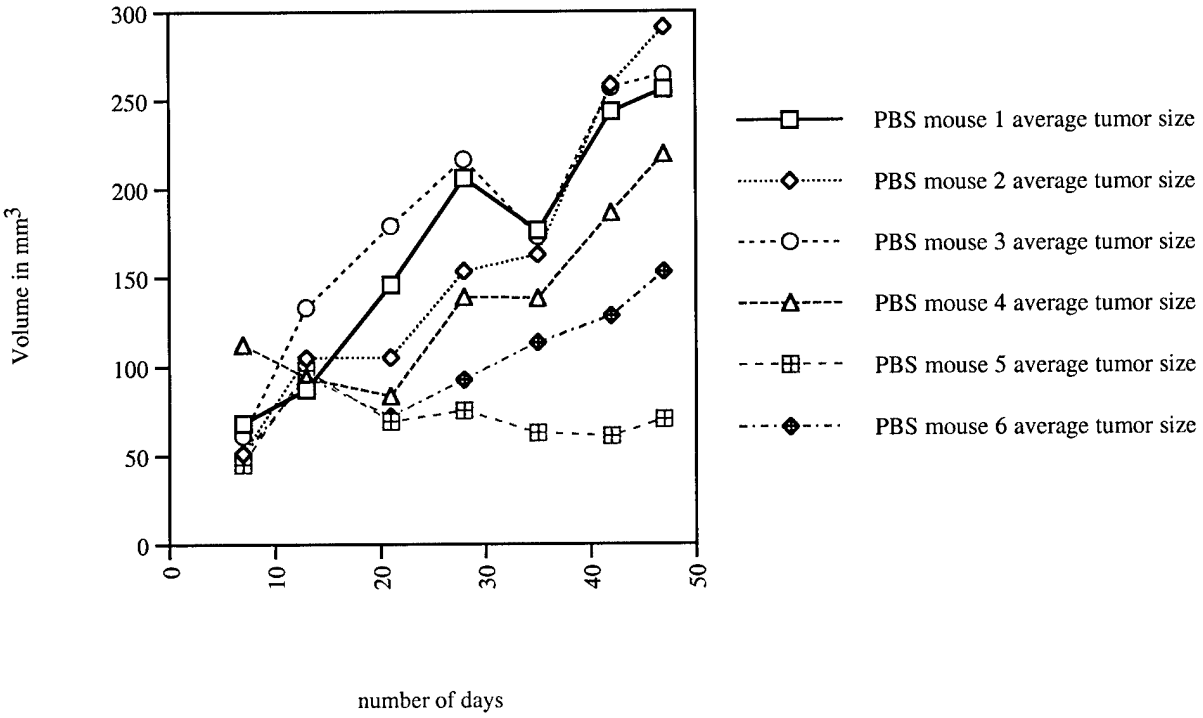


**Figure 5. Comparison of Average T47D-DE Tumor Sizes in PBS-Injected and 5-Aza-Cdr-Injected Nude Mice.**

The data from Figure 3 and Figure 4 were consolidated onto one graph for direct comparison. The average tumor sizes in $mm^3$ for all of the six mice in the PBS-injected control mice and the average of the two 5-Aza-Cdr-injected experimental mice are plotted against time in number of days. Again, the tumors were allowed to grow for 4 and 1/2 weeks (33 days) prior to starting the injections of either PBS or 5-Aza-Cdr.

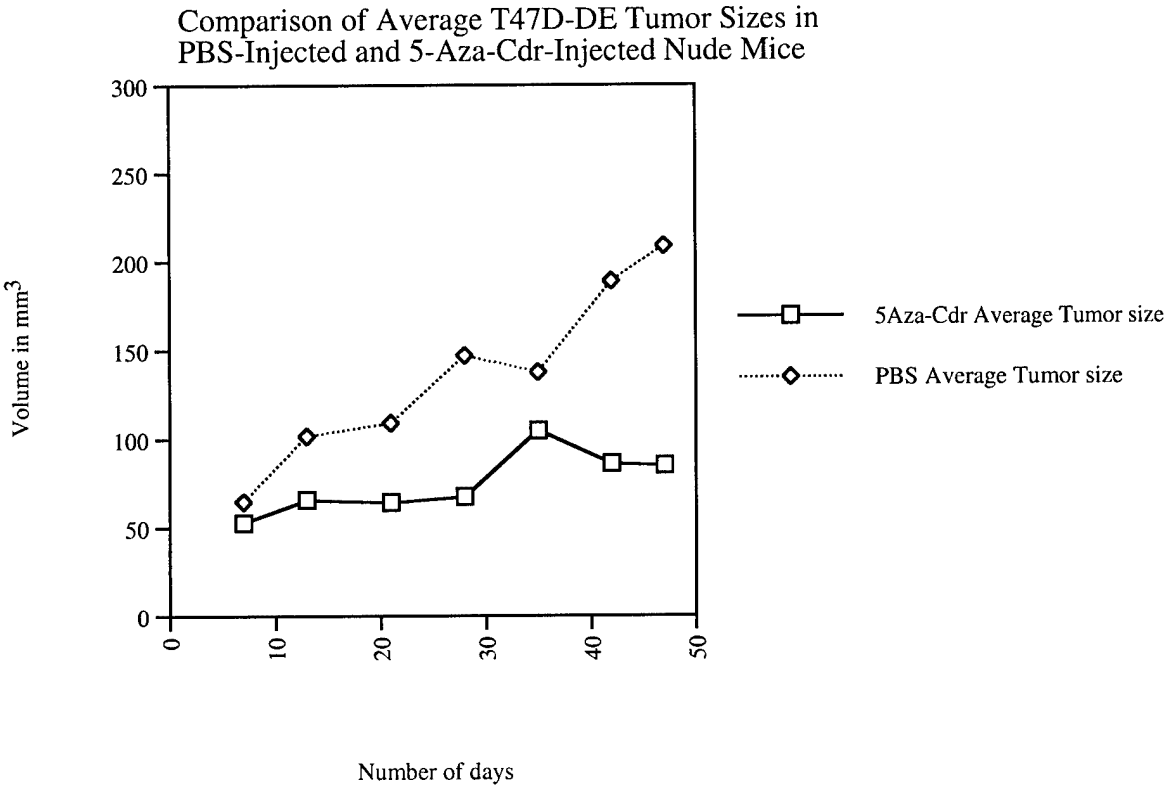

Comparison of Average T47D-DE Tumor Sizes in PBS-Injected and 5-Aza-Cdr-Injected Nude Mice
Volume in mm3
300
250
200
150
100
50
0
0
10
20
30
40
50
Number of days
5Aza-Cdr Average Tumor size
PBS Average Tumor size

**Figure 6. Methylation-specific PCR of Breast Cancer Patient Samples Demonstrate Variability Problems.**

10 ml of breast cancer patient plasma (previously frozen at –80°C) were thawed then heated to 95°C for 5 minutes. Following centrifugation at 14K rpm, DNA was extracted from the supernatant using a QIAamp blood and tissue kit (Qiagen). DNA was subjected to methylation-specific PCR protocol using primers specific to methylated (M) and unmethylated (UM) p16 promoter sequences. Lanes labeled 'Blank' contained all of the PCR components plus $H_2O$ instead of DNA template, to negatively control for contamination. PCR reactions were also carried out for samples derived from the breast cancer cell line T47D-DE, which shows PCR signals for both M and U p16 promoter sequences. The date at the side of each sub-panel shows when the particular PCR reaction took place.

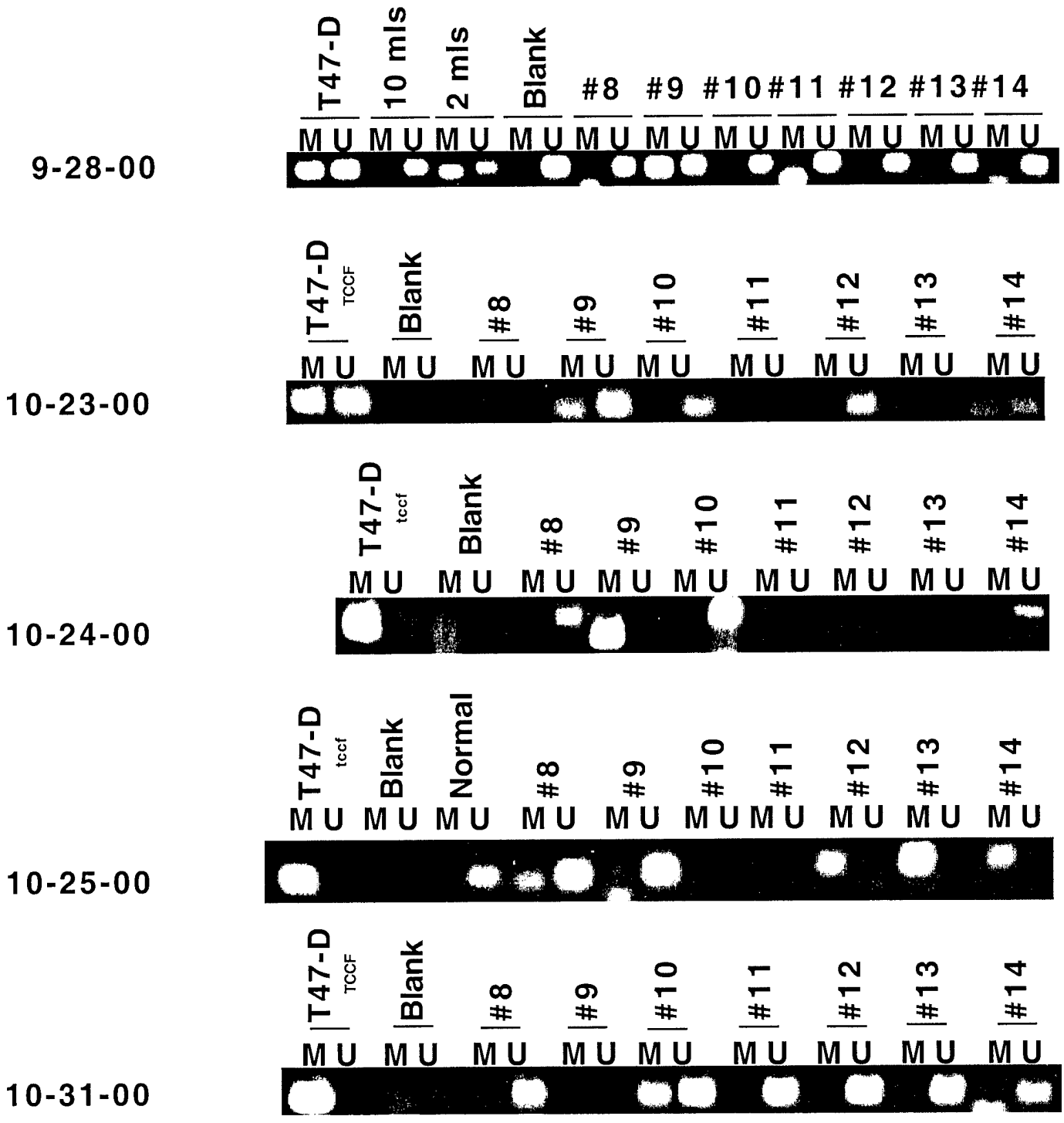
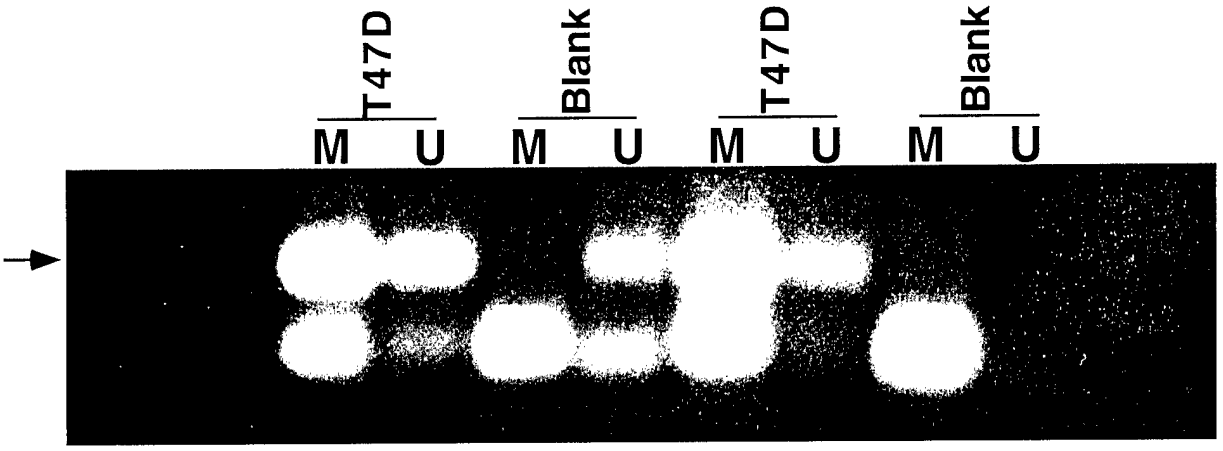
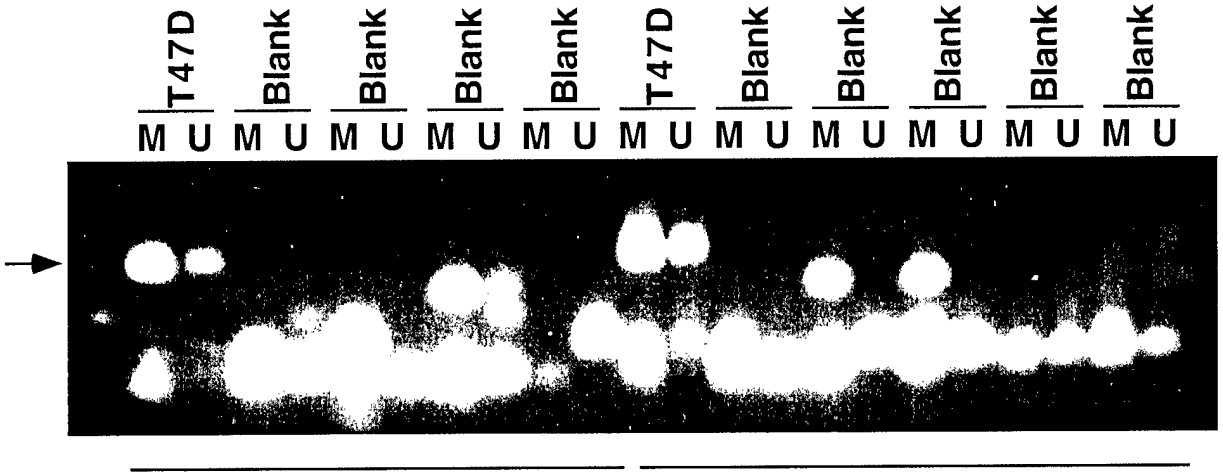

9-28-00
T47-D
10 mls
2 mls
Blank
#8
#9
#10
#11
#12
#13
#14
M U
10-23-00
T47-D TCCF
Blank
#8
#9
#10
#11
#12
#13
#14
10-24-00
T47-D tccf
Blank
#8
#9
#10
#11
#12
#13
#14
10-25-00
T47-D tccf
Blank
Normal
#8
#9
#10
#11
#12
#13
#14
10-31-00
T47-D TCCF
Blank
#8
#9
#10
#11
#12
#13
#14

**Figure 7. Methylation-specific PCR control reactions show contamination problems:** DNA was isolated from Breast Cancer Cell Line T47D and subjected to methylation-specific PCR along with blank controls that contain purified $H_2O$ instead of DNA template. The arrows designate where the PCR product for methylated (M) and unmethylated (U) products should run on each gel. The T47D DNA demonstrates PCR signals from both methlylated and unmethylated specific primers. The upper panel also demonstrates a contamination problem. It is surprising to find signals for unmethylated DNA in the blank controls that do not contain any DNA template. The bottom panel shows multiple reactions that were set up either in a flow hood or on the laboratory bench to determine if contamination was specific to an area in the laboratory that might contain contaminating template DNA. Again signals for Methylated and Unmethylated DNA were seen in both the T47D and $H_2O$ blank controls.




Hood

Bench

Same solutions used for
all PCR rxn

**Figure 8. Methylation-specific PCR analysis of blood plasma from Breast Cancer Patients at the p16 locus.**

10ml of Breast Cancer patient plasma (previously frozen at –80$^{o}$C) were thawed then heated to 99$^{o}$C for 5 minutes. Following centrifugation at 14K rpm, DNA was extracted from the supernatant using a QIAamp blood and tissue kit (Qiagen). DNA was subjected to Sodium bisulfite modification followed by a modified two-stage methlyation-specific PCR protocol using primers to methylated (M), and unmethylated (U) p16 promoter sequences. In addition, three controls were performed. Lanes labeled 'Blank' contained all of the PCR components plus $H_2O$ instead of DNA template, to negatively control for contamination. DNA from cell line HBL100 was either subjected to both SssI methylase or not to generate positive controls for the methylated or unmethylated MS-PCR reactions, respectively. Patient numbers are indicated above each set of reactions. This particular experiment shows a representative sample of some of the patients in our study. Of note, patient #33 shows promoter methylation of the p16 locus.

HBL 100 M
HBL 100 UM
Blank
#7
#8
#9
#10
M U M U M U M U M U M U M U


HBL 100 M
HBL 100 UM
Blank
#15
#16
#17
#18
#19
M U M U M U M U M U M U M U M U


HBL 100 M
HBL 100 UM
Blank
#30
#33
M U M U M U M U M U

| Breast Cancer Cell Line | Rb expression | p16 expression | MS-PCR | Nude Mice |
|---|---|---|---|---|
| HCC 38 | + | - | UM | n/a |
| HCC 70 | + | + | n/d | n/a |
| HCC 202 | + | - | M | n/a |
| HCC 1187 | + (weak) | - | n/d | n/a |
| HCC 1395 (first) | + | - | p16 deleted | n/a |
| HCC 1395 (second) | + | - | UM | n/a |
| HCC 1419 | + | - | UM | n/a |
| HCC 1428 | + (weak) | - | M + UM | n/a |
| HCC 1569 | + | + | n/d | n/a |
| HCC 1599 | + | - | p16 deleted | n/a |
| HCC 1806 | + | - | UM | n/a |
| HCC 1937 | - | + (weak) | n/d | n/a |
| HCC 1954 | - | - | n/d | n/a |
| HCC 2157 | + | + | n/d | n/a |
| HCC 2218 | + | n/d | n/d | n/a |
| BT 20 | + (weak) | - | p16 deleted | n/a |
| SKBR-3 | + | - | UM | n/a |
| CAMA-1 | + | - | UM | n/a |
| HS 578 BST | + | + | n/d | n/a |
| MDA-MB-134VI | + | - | M | 1 died<br>2 no tumors<br>2 tumors |

**Table 1. Screening of Breast Cancer Cell Lines for Rb (+) expression and p16 (-) expression due to promoter methylation for use in tumorigenecity assays.**
Breast Cancer Cell Lines were grown in cell culture and subjected to the following assays: Immunoblot analysis was performed on protein extracts from above list of cell lines for both Rb and p16. Cell lines that exhibited both strong positive Rb expression and negative p16 expression, were further subjected to methylation specific PCR to determine if lack of expression of p16 was due to promoter methylation at the DNA level. Methylated alleles are designated (M) while unmethylated alleles are designated (UM). It was determined that two cell lines exhibited these criteria: HCC202 and MBA-MB-134 VI. Only MBA-MB-134 VI was chosen for tumorigenecity assays as HCC 202 grew too slowly in cell culture to generate enough cells for injections into nude mice. Results of the tumor studies are indicated in the last column.

**Table 2. List of Different Cell Culture Media Requirements for Individual Breast Cancer and Normal Cell Lines.**

| MEDIUM | | CELL LINE | SPLIT |
|---|---|---|---|
| RPMI Bottle | 500 ML | HCC 1187 | 1:2 |
| Glucose (stock 25g/L) | 5.6ML | HCC 1599 | 1:2 to 1:4 |
| Hepes | 5.6ML | | |
| Sodium Pyruvate | 5.6ML | HCC 1954 | 1:3 to 1:4 |
| Fetal Bovine Serum | 56ML | | |
| Gibco BRL Cat# 11-875-093 | | HCC 1806 | 1:2 to 1:4 |
| | | HCC 1395 | 1:2 |
| | | HCC 1428 | 1:4 to 1:8 |
| | | HCC 70 | 1:4 to 1:6 |
| | | HCC 38 | 1:2 to 1:4 |
| | | HCC 202 | 1:2 |
| | | HCC 1419 | 1:2 |
| | | HCC 1569 | 1:2 to 1:4 |
| | | HCC 1937 | 1:2 |
| | | HCC 2157 | 1:2 |
| | | HCC 2218 | 1:2 |
| Minimum Essential Medium (MEM) | 500 ML | BT 20 | 1:3 |
| Fetal Bovine Serum | 56ML | | |
| Pen/Strep/Glut | 5.6 ML | CAMA 1 | 1:3 |
| Non essential amino acids | 5.6 ML | | |
| Sodium Pyruvate | 5.6 ML | | |
| Gibco BRL Cat# 11-095-080 | | | |
| Modified delbecco's medium (DMEM) | 500 ml | HS 578T | 1:3 to 1:8 |
| Fetal Bovine Serum | 56ML | T47D (DE) | 1:4 to 1:6 |
| Pen/Strep/Glut | 5.6 ML | | |
| EGF | 30 ng/ML | ZR75.1 | 1:4 to 1:6 |
| Gibco BRL Cat# 11-965-092 | | | |
| Minimum Essential Medium (MEM) | 500 ML | MDA-MB-231 | 1:2 to 1:4 |
| Fetal Bovine Serum | 56ML | MDA-MB-361 | 1:2 to 1:6 |
| Pen/Strep/Glut | 5.6 ML | MCF-7 | 1:4 to 1:8 |
| Gibco BRL Cat# 11-095-080 | | HBL 100 | 1:4 to 1:8 |
| RPMI | 500ML | DU4475 | 1:2 to 1:4 |
| Fetal Bovine Serum | 56ML | | |
| Pen/Strep/Glut | 5.6 ML | | |
| Gibco BRL Cat# 11-875-093 | | | |
| McCoyes 5a | 500ML | SK-BR-3 | 1:2 |
| Fetal Bovine Serum | 56ML | | |
| Pen/Strep/Glut | 5.6 ML | | |
| Irvine Scintific(800-437-5706) Cat # 9090 | | | |

# COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD

Office of the COMIRB

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P.O. Box 6508
Campus box F-490
Aurora, Colorado 80045-0508
(303) 724-1055
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10/13/2000

Assurance # M-1494-01

Participating Institutions

The Children's Hospital
Colorado Prevention Center
Denver Health and Hospitals
University of Colorado Health Sciences Center
Department of Veterans Affairs Medical Center, Denver
University of Colorado Hospital

## Certificate of Approval

**Investigator:** ANDREW KRAFT

**Sponsor:** ARMY CTR AWARD

Primary Contact:
KRAFT, ANDREW
Campus Box B171

**COMIRB Protocol 00-848, Initial Review -> Expedited Status**

**Protocol Date:** 09/06/2000

**Approval Date:** **10/12/2000**

**Approval Expires:** **10/11/2001**

**Title:**

ESTIMATION OF ABNORMAL BREAST CANCER DNA IN PATIENT'S BLOOD USING A SENSITIVE PCR BASED ASSAY

**Approval Includes:**

**Protocol**
**Investigator**
**Consent and/or Assent Form**

**All COMIRB Approved Investigators must comply with the following:**

- For the duration of your protocol, any change in the experimental design/consent and/or assent form must be approved by the COMIRB before implementation of the changes.
- Use only a copy of the **COMIRB signed and dated Consent and/or Assent Form.** The investigator bears the responsibility for obtaining from all subjects "Informed Consent" as approved by the COMIRB. The COMIRB REQUIRES that the subject be given a signed copy of the consent and/or assent form. Consent and/or assent forms must include the name and telephone number of the investigator.
- **Provide non-English speaking subjects with a certified translation of the approved Consent and/or Assent Form** in the subject's first language. A copy of the translator's certification should be attached to the consent and/or form.
- The Investigator also bears the responsibility **for informing the COMIRB immediately of any Serious Adverse Events** (deaths, serious complications or other untoward effects of this research at this or other sites), and of the relationship of the SAE to the investigational trial. The COMIRB uses the standard definition of Serious or Unanticipated Events that include: death, hospitalization or prolongation of hospitalization and other unanticipated side effects.
- Obtain COMIRB **approval for all advertisements** before use.
- **Federal regulations require a Continuing Review to renew approval of this project** within a 12-month period from the last approval date unless otherwise indicated in the review cycle listed below. If you have a restricted/high risk protocol, specific details will be outlined on this letter under the heading "COMIRB approval is granted subject to the following criteria". Non-compliance with Continuing Review will result in the termination of this study. This project has been assigned the following review cycle:

**COMIRB Continuing Review Cycle: 12 months**

We will send you a Continuing Review Form to be completed prior to the due date.

Any questions regarding the COMIRB action of this study should be referred to the COMIRB staff at 303-724-1055 or UCHSC Box F-490.

10/12/00

Christopher Kuni, MD Chair
Ken Easterday, RPh Co-Chair

Allan V. Prochazka, MD, MSc Chair
Stephen Bartlett, RPh Co-Chair

Adam Rosenberg, MD Chair
Dave Lawellin, PhD Co-Chair

Consent Form Approval

Allan Prochazka, MD/Stephen Bartlett, R.Ph., Co-Chairs, COMIRB
Christopher Kuni, MD/Ken Easterday, R.Ph., Co-Chairs, COMIRB
Adam Rosenberg, MD/David Lawellin, Ph.D..,Co-Chairs, COMIRB

Date: 10/2/00 Valid Through: 10/1/01

**COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD**

**"Estimation of abnormal breast cancer DNA in patient's blood using a sensitive PCR based assay"**

**Principal Investigator: Andrew S. Kraft**
**SUBJECT CONSENT FORM**
**September 6, 2000/COMIRB Protocol Number 00-848**

## Project Description

You are being asked to take part in an investigational study evaluating the use of a blood assay to measure the levels of a specific modified piece of DNA in your blood. Breast cancer is caused in part by the lack of the ability of cells to produce specific proteins that inhibit the growth of cells. This lack of protein production is caused by modification of cellular DNA. This DNA abnormality is not an inherited trait and the reason it occurs is not known. Since tumor cells are growing and dying some of this abnormal DNA may be released into the blood. Using a newly developed laboratory assay, we would like to attempt to measure this abnormal DNA in your blood. One hundred women with metastatic breast cancer will be enrolled into this study. Participation in this study is voluntary and the purpose of this consent is to inform you about the study and its possible benefits and risks.

## Procedures

If you agree to participate, you will need to donate a single 20ml of blood (two tablespoons) at a time when you are known to have breast cancer. This blood will be sent to the lab for evaluation of abnormal DNA levels. The principal investigator or a designee will review your chart in order to identify information regarding the stage of your breast cancer.

Initials _____

## Discomforts and Risks

### Venipuncture Risk
Approximately 2 tablespoon of blood (20ml) will be removed by putting a needle into your vein at the time of your pre-operative evaluation. This is the standard method used to obtain blood for tests. You will feel pain when the needle goes into the vein. A bruise may form at the site.

### Benefits
You will receive no benefit from participating in this research study and there are risks as mentioned in the risk section.

### Source of Funding
All funding for this study will be provided by the Army Medical Research Command.

### Cost to Subject
There is no cost to you for participating in this study. You will not be paid for your participating in this study.

### Study Withdrawal
You may choose not to enter the study or withdraw from the study at any time and your doctor will continue to take care of you without loss of benefits to which you are entitled. Significant new findings that relate to your participation in this study will be discussed with you.

### Invitation for Questions
You will receive a copy of this consent form. Please ask questions about this research or consent either now or in the future. You may direct your questions to Dr. Andrew S. Kraft, MD 303-315-8802. If you have questions regarding your rights as a research subject, please call the Colorado Multiple Institutional Review Board (COMIRB) office at (303) 724-1055.

### Confidentiality
Your physician/investigator and the Army Breast Cancer Research Command will treat your identity with professional standards of confidentiality. However, the U.S. Department of Health and Human Services, and the Colorado Multiple Institute Review Board have the right to inspect all of your medical records relating to this research for the purpose for verifying data. The information obtained in this study may be published in medical journals, but your identity will not be revealed.

### Injury and Compensation
If you are hurt by this research, we will provide medical care if you want it, but you will have to pay for the care that is needed. You will not be paid for any other loss as a result of the injury, such as loss of wages, pain and suffering. Further information can be obtained by calling Andrew S. Kraft, MD 303-315-8802.

Initials _____

**AUTHORIZATION:**

I have read this paper about the study or it was read to me. I know what will happen, both the possible good and bad (benefits and risks). I choose to be in this study. I know I can stop being in this study and I will still get the usual medical care. I will get a copy of this consent form. (Initial all the previous pages of this consent form)

Signature: _____ Print Name _____ Date _____
Subject

Consent form explained by: _____ Print Name _____ Date _____

Investigator _____ Date _____

Initials _____

# COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD

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Aurora, Colorado 80045-0508
(303) 724-1055
(303) 724-0990 (Fax)
10/13/2000

The Children's Hospital
Colorado Prevention Center
Denver Health and Hospitals
University of Colorado Health Sciences Center
Department of Veterans Affairs Medical Center, Denver
University of Colorado Hospital

## Certificate of Approval

**Investigator:** ANDREW KRAFT

**Sponsor:** ARMY CTR AWARD

Primary Contact:
KRAFT, ANDREW
Campus Box B171

**COMIRB Protocol 00-849, Initial Review -> Expedited Status**

**Protocol Date:** 09/06/2000

**Approval Date:** **10/12/2000**

**Approval Expires:** **10/11/2001**

**Title:**

CORRELATION BETWEEN METHYLATED P16 DNA IN THE BLOOD AND BREAST CANCER TISSUE OF PATIENTS

**Approval Includes:**

**Protocol**
**Investigator**
**Consent and/or Assent Form**

**All COMIRB Approved Investigators must comply with the following:**

- For the duration of your protocol, any change in the experimental design/consent and/or assent form must be approved by the COMIRB before implementation of the changes.
- Use only a copy of the **COMIRB signed and dated Consent and/or Assent Form**. The investigator bears the responsibility for obtaining from all subjects "Informed Consent" as approved by the COMIRB. The COMIRB REQUIRES that the subject be given a signed copy of the consent and/or assent form. Consent and/or assent forms must include the name and telephone number of the investigator.
- **Provide non-English speaking subjects with a certified translation of the approved Consent and/or Assent Form** in the subject's first language. A copy of the translator's certification should be attached to the consent and/or form.
- The Investigator also bears the responsibility **for informing the COMIRB immediately of any Serious Adverse Events** (deaths, serious complications or other untoward effects of this research at this or other sites), and of the relationship of the SAE to the investigational trial. The COMIRB uses the standard definition of Serious or Unanticipated Events that include: death, hospitalization or prolongation of hospitalization and other unanticipated side effects.
- Obtain COMIRB **approval for all advertisements** before use.
- **Federal regulations require a Continuing Review to renew approval of this project** within a 12-month period from the last approval date unless otherwise indicated in the review cycle listed below. If you have a restricted/high risk protocol, specific details will be outlined on this letter under the heading "COMIRB approval is granted subject to the following criteria". Non-compliance with Continuing Review will result in the termination of this study. This project has been assigned the following review cycle:

**COMIRB Continuing Review Cycle: 12 months**

We will send you a Continuing Review Form to be completed prior to the due date.

Any questions regarding the COMIRB action of this study should be referred to the COMIRB staff at 303-724-1055 or UCHSC Box F-490.

10/12/00

Consent Form Approval

Allan Prochazka, MD/Stephen Barnett, R.Ph., Co-Chairs, COMIRB
Christopher ~~Kuni, MD/Ken Easterday~~, R.Ph., Co-Chairs, COMIRB
Adam Rosenberg, MD/David Lawellin, Ph.D...Co-Chairs, COMIRB

Date: 10/12/00 Valid Through: 10/11/01

**COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD**

**"Correlation between methylated p16 DNA in the blood and breast cancer tissue of patients"**

**Principal Investigator: Andrew S. Kraft**
**SUBJECT CONSENT FORM**
**September 6, 2000/COMIRB Protocol Number 00-849**

## Project Description

You are being asked to take part in an investigational study evaluating the use of a blood assay to measure the levels of a specific modified piece of DNA in your blood. Breast cancer is caused in part by the lack of the ability of cells to produce specific proteins that inhibit the growth of cells. This lack of protein production is caused by modification of cellular DNA. This DNA abnormality is not an inherited trait and the reason it occurs is not known. Since tumor cells are growing and dying some of this abnormal DNA may be released into the blood. One hundred women will be enrolled into this study with 20 women each having Stage II, III or IV breast carcinoma. This study will identify whether the abnormal DNA found in your breast cancer is also found in your blood. Participating in this study does not mean that you have any hereditary abnormality in your DNA. This study will measure the levels of abnormal DNA found in your tumor and attempt to correlate the results of this blood test with the tumor sample. Participation in this study is voluntary and the purpose of this consent is to inform you about the study and its possible benefits and risks.

## Procedures

If you agree to participate, you will need to donate 20 ml of blood (two tablespoons), at the time of your pre-operative evaluation. This blood will be sent to the lab for evaluation of free DNA. During your surgery your doctor will be removing tissue for clinical evaluation. When the pathologic evaluation is complete, we will take the unused tissue for further study. The tissue to be used for this study would normally be discarded. No additional tissue will be taken at the time of operation The findings in the research lab will then be correlated with the results of the blood test. You will not

Initials _____

receive any information regarding these additional tests. The principal investigator or a designee will review your chart in order to identify information regarding the stage of your breast cancer.

## Discomforts and Risks

### Breast Biopsy

There will be no additional risk to you other than the usual risks for your surgery. This study will not involve any additional operative procedures. No additional risks are associated with this research since the tissue being used for clinical purposes only.

### Venipuncture Risk

Approximately 1 tablespoon of blood (20 ml) will be removed by putting a needle into your vein at the time of your pre-operative evaluation. This is the standard method used to obtain blood for tests. You will feel pain when the needle goes into the vein. A bruise may form at the site.

## Benefits

You will receive no benefit from participating in this research study and there are risks as mentioned in the risk section.

## Source of Funding

All funding for this study will be provided by the Army Breast Cancer Research Command.

## Cost to Subject

There is no cost to you for participating in this study. There will be no charge for procedures or labs required by the study. You will not be paid for your participating in this study.

## Study Withdrawal

You may choose not to enter the study or withdraw from the study at any time and your doctor will continue to take care of you without loss of benefits to which you are entitled. Significant new findings that relate to your participation in this study will be discussed with you.

## Invitation for Questions

You will receive a copy of this consent form. Please ask questions about this research or consent either now or in the future. You may direct your questions to Dr. Kraft at (303) 315-8802. If you have questions regarding your rights as a research subject, please call the Colorado Multiple Institutional Review Board (COMIRB) office at (303) 724-1055.

## Confidentiality

Your physician/investigator and the Army Breast Cancer Research Command will treat your identity with professional standards of confidentiality. However, the U.S. Department of health and Human Services, the Army Research Command and the

Initials _____

Colorado Multiple Institute Review Board have the right to inspect all of your medical records relating to this research for the purpose for verifying data. The information obtained in this study may be published in medical journals, but your identity will not be revealed.

**Injury and Compensation**
If you are hurt by this research, we will provide medical care if you want it, but you will have to pay for the care that is needed. You will not be paid for any other loss as a result of the injury, such as loss of wages, pain and suffering. Further information can be obtained by calling Andrew S. Kraft, MD 303-315-8802.

**AUTHORIZATION:**
I have read this paper about the study or it was read to me. I know what will happen, both the possible good and bad (benefits and risks). I choose to be in this study. I know I can stop being in this study and I will still get the usual medical care. I will get a copy of this consent form. (Initial all the previous pages of this consent form)

Signature: _____ Print Name _____ Date _____
Subject

Consent form explained by: _____ Print Name _____ Date _____

Investigator _____ Date _____

Initials _____